REMARKS

Claims 109-120 are pending and claims 1-108 and 121-171 have been canceled without prejudice. Applicants expressly reserve the right to pursue the canceled subject matter in this application or subsequent applications that claim the benefit of this application.

Applicants note that eculizumab is h5G1.1-mAb, it is not h5G1.1-scFv as the Examiner stated in the 35 U.S.C. § 103(a) rejection in the Office Action of March 21, 2008. Rather h5G1.1-scFv is the single chain antibody named pexelizumab.

Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

DETAILED ACTION

Continued Examination

1. Applicant notes with appreciation that the amendment filed January 10, 2008 has been entered. The Examiner has acknowledged that no outstanding grounds for rejection are maintained.

Claim Rejections under 35 U.S.C. § 103

2. The Examiner has rejected claims 109-120 under 35 U.S.C. § 103(a) as allegedly being upatentable over Alexion's press release dated January 6, 2003 in view of Collard et al. (Arterioscler Thromb Vasc Biol. [1999] 19(11): 2623-29). The Examiner alleges that Alexion's press release teaches the use of the anti-C5 antibody, eculizumab, for the treatment of subjects with paroxysmal nocturnal hemoglobinuria (PNH). The Examiner alleges that treatment of NO deficiency in PNH patients would be an inherent property of eculizumab. The Examiner states that the press release does not specifically link the hemolytic disease with NO deficiency or the effect of h5G1.1-scFv on NO levels. The Examiner alleges, "Collard teaches the treatment of hypoxic HUVECs with h5G1.1-scFv. Collard teaches that terminal complement component C5b-9 deposition results in a functional loss of NO-dependent relaxation, increases VCAM-1 expression and decreases cGMP levels. Collard teaches that decreased cGMP levels may compromise vascular blood flow because

of decreased endothelium-dependent relaxation and increased adhesion of neutrophils to the endothelium. Collard teaches that h5G1.1-scFv treatment of the HUVECs attenuates C5b-9 deposition and preserves acetylcholine induced increases in cGMP after hypoxia/reoxygenation." The Examiner alleges it would have been obvious to use h5G1.1-scFv antibody for the treatment of NO deficiency in a subject. Applicants respectfully traverse.

To negate the patentability of the claimed invention, the cited combination of references must teach or suggest each and every element of the claimed invention. The clinical study in the cited Alexion press release was specific to treating PNH patients. The press release does not disclose that all patient populations could be treated for NO deficiency with compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components as is claimed in the present application. The teachings and observations of the press release are limited to PNH patients.

The deficiencies of the press release are not remedied by the teachings of Collard et al. Collard et al. treat hypoxic/reoxygenated HUVEC cells with anti-C5 antibodies thereby attenuating C5b-9 deposition and relieving a functional loss of NO-dependent relaxation through a mechanism involving decreased cGMP signaling. Collard et al. do not disclose that NO deficiency can be treated with complement inhibitors as claimed in the present application. Relieving a functional loss of NO-dependent relaxation does not suggest treatment of NO deficiency in all situations. Functional loss of NO-dependent relaxation is not equivalent to NO deficiency as the latter occurs upstream of the decreased cGMP signaling shown in Collard et al. For example, NO deficiency results in reduced clot dissolution and significant fibrin deposition and thrombus formation through direct interactions of NO not involving cGMP signaling (see Exhibit A; page 1658, center column). One of ordinary skill in the art would not expect the teachings of Collard et al. to relieve symptoms of NO deficiency not associated with decreased cGMP signaling.

In addition, the disclosure of Collard et al. is limited to situations where cells are reoxygenated after hypoxia. One of ordinary skill in the art would not expect that anti-C5 antibodies would relieve a functional loss of NO-dependent relaxation under all circumstances based on the teaching of Collard et al.

Given that the press release and Collard et al. fail to teach or suggest each and every element of the claimed invention, the combination of references fails to render the claimed invention obvious. Reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. If an additional fee is due, please charge our Deposit Account No. 18-1945, under Order No. **ALXN-P01-114** from which the undersigned is authorized to draw.

Dated: June 23, 2008

Respectfully submitted,

By _____

Ryan Murphey
Registration No.: 61,156
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

Exhibit A

The Clinical Sequelae of Intravascular Hemolysis and Extracellular Plasma Hemoglobin

A Novel Mechanism of Human Disease

Russell P. Rother, PhD
Leonard Bell, MD
Peter Hillmen, MB, PhD
Mark T. Gladwin, MD

Context The efficient sequestration of hemoglobin by the red blood cell membrane and the presence of multiple hemoglobin clearance mechanisms suggest a critical need to prevent the buildup of this molecule in the plasma. A growing list of clinical manifestations attributed to hemoglobin release in a variety of acquired and iatrogenic hemolytic disorders suggests that hemolysis and hemoglobinemia should be considered as a novel mechanism of human disease.

Evidence Acquisition Pertinent scientific literature databases and references were searched through October 2004 using terms that encompassed various aspects of hemolysis, hemoglobin preparations, clinical symptoms associated with plasma hemoglobin, nitric oxide in hemolysis, anemia, pulmonary hypertension, paroxysmal nocturnal hemoglobinuria, and sickle-cell disease.

Evidence Synthesis Hemoglobin is released into the plasma from the erythrocyte during intravascular hemolysis in hereditary, acquired, and iatrogenic hemolytic conditions. When the capacity of protective hemoglobin-scavenging mechanisms has been saturated, levels of cell-free hemoglobin increase in the plasma, resulting in the consumption of nitric oxide and clinical sequelae. Nitric oxide plays a major role in vascular homeostasis and has been shown to be a critical regulator of basal and stress-mediated smooth muscle relaxation and vasomotor tone, endothelial adhesion molecule expression, and platelet activation and aggregation. Thus, clinical consequences of excessive cell-free plasma hemoglobin levels during intravascular hemolysis or the administration of hemoglobin preparations include dystonias involving the gastrointestinal, cardiovascular, pulmonary, and urogenital systems, as well as clotting disorders. Many of the clinical sequelae of intravascular hemolysis in a prototypic hemolytic disease, paroxysmal nocturnal hemoglobinuria, are readily explained by hemoglobin-mediated nitric oxide scavenging.

Conclusion A growing body of evidence supports the existence of a novel mechanism of human disease, namely, hemolysis-associated smooth muscle dystonia, vasculopathy, and endothelial dysfunction.

JAMA. 2005;293:1653-1662 www.jama.com

HEMOGLOBIN IS A HIGHLY CONserved molecule found in species ranging from single-cell organisms to mammals, but the role of hemoglobin in different organisms varies. While hemoglobin in bacteria functions as a nitric oxide sump by oxidation of nitric oxide to nitrate,[1-3] hemoglobin functions to remove oxygen in nematodes, a critical task for anaerobes.[4] By contrast, in mammals, hemoglobin primarily serves a respiratory function in the delivery of oxygen and removal of carbon dioxide. Based on the recent discovery that nitric oxide is a critical regulator of vasodilation and vascular homeostasis, the interactions of nitric oxide with hemoglobin in mammals has drawn increasing interest. Because the reaction of nitric oxide with the vast amounts of intravascular oxyhemoglobin (16 g/dL) is fast (10^7 $M^{-1}s^{-1}$) and irreversible, it would be expected that nitric oxide produced by endothelium would be immediately scavenged by hemoglobin and would therefore be incapable of paracrine diffusion from endothelium to vascular smooth muscle.[5,6] However, the ability of hemoglobin to react with nitric oxide produced by endothelium is limited by compartmentalization of hemoglobin inside the erythrocyte.[7-9] Thus, the evolution of the erythrocyte may be considered as a mechanism of reducing toxicity while

CME available online at www.jama.com

Author Affiliations: Alexion Pharmaceuticals, Cheshire, Conn (Drs Rother and Bell); Department of Haematology, Leeds Teaching Hospitals NHS Trust, Leeds, England (Dr Hillmen); Vascular Therapeutics Section, Cardiovascular Branch, National Heart, Lung, and Blood Institute and Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Md (Dr Gladwin).
Corresponding Authors: Russell P. Rother, PhD, Vice President of Research, Alexion Pharmaceuticals, 352 Knotter Dr, Cheshire, CT 06410 (rotherr@alxn.com); Mark T. Gladwin, MD, Vascular Therapeutics Section, Cardiovascular Branch, NHLBI, Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bldg 10-CRC, Room 5-5140, 10 Center Dr, MSC 1454, Bethesda, MD 20892-1454 (mgladwin@nih.gov).
Clinical Review Section Editor: Michael S. Lauer, MD. We encourage authors to submit papers for consideration as a "Clinical Review." Please contact Michael S. Lauer, MD, at lauerm@ccf.org.

ensuring separation of the critical respiratory transport machinery needed for efficient oxygen delivery from the endothelium. Moreover, multiple systems have evolved to control the level of cell-free hemoglobin in the plasma during normal physiological hemolysis, presumably to curtail the deleterious effects of plasma hemoglobin on nitric oxide bioavailability and endothelial function.

During intravascular hemolysis, cell-free plasma hemoglobin may overwhelm homeostatic systems in place to remove it.[10] Hemolytic conditions with substantial intravascular hemolysis include paroxysmal nocturnal hemoglobinuria (PNH), sickle-cell disease (SCD), thalassemias, hereditary spherocytosis and stomatocytosis, microangiopathic hemolytic anemias, pyruvate kinase deficiency, ABO mismatch transfusion reaction, paroxysmal cold hemoglobinuria, severe idiopathic autoimmune hemolytic anemia, infection-induced anemia, malaria, cardiopulmonary bypass, mechanical heart valve–induced anemia, and chemical-induced anemias. Although the various hemolytic diseases each have unique symptoms, they often share hemoglobinemia-related sequelae. In addition, observations from the clinical administration of artificial, purified, and recombinant hemoglobin solutions have provided further support for the causal relationship between excess cell-free hemoglobin in the bloodstream, symptoms, and cardiovascular events.[11-18]

Nitric oxide scavenging by excess plasma hemoglobin has been implicated in various clinical manifestations of intravascular hemolysis. Nitric oxide is a regulator of smooth muscle tone and platelet activation, and reductions in nitric oxide plasma levels lead to smooth muscle dystonias, including hypertension, gastrointestinal contractions, and erectile dysfunction, as well as clot formation.[16,17,19-26] Hemoglobin also exerts direct cytotoxic, inflammatory, and pro-oxidant effects that adversely affect endothelial function.[27]

EVIDENCE ACQUISITION

Multiple searches were performed with the New England Research Application Center and PubMed through October 2004, including various combinations of the following key search terms: *hemoglobin*, *recombinant*, *cell-free*, *stroma-free*, *artificial*, *heme*, *hemolysis*, *marker*, *lactate dehydrogenase*, *LDH*, *anemia*, *pulmonary hypertension*, *abdominal*, *abdomen*, *pain*, *erectile dysfunction*, *dysphagia*, *smooth muscle*, *nitric oxide*, *paroxysmal nocturnal hemoglobinuria*, and *sickle-cell disease*. References cited in textbooks or articles were also used in some cases. Plasma hemoglobin concentrations are reported throughout this review in terms of hemoglobin tetramer. Because each tetramer contains 4 heme groups, hemoglobin can react with and inactivate 4 nitric oxide molecules.

EVIDENCE SYNTHESIS

The Removal of Hemoglobin During Intravascular Hemolysis

When red blood cells (RBCs) are destroyed within the vascular compartment, hemoglobin escapes into the plasma, dimerizes, and is rapidly bound by the serum protein haptoglobin. The haptoglobin-hemoglobin complex exposes a neoepitope that is recognized by the hemoglobin scavenger receptor, CD163 on the surface of monocytes/macrophages, which binds the complex with high affinity and mediates haptoglobin-hemoglobin endocytosis and degradation.[28,29] Since haptoglobin is not recycled, formation of large amounts of haptoglobin-hemoglobin complexes leads to rapid haptoglobin depletion. Thus, in severe hemolytic diseases such as PNH and SCD, serum haptoglobin is typically undetectable.[10]

Ferrous heme (Fe^{II}), the oxygen-binding component of hemoglobin, can be oxidized to ferric heme (Fe^{III}), which is then released from hemoglobin and binds with high affinity to a plasma glycoprotein, hemopexin. Heme bound to hemopexin is degraded in a series of enzymatic steps in the liver. Heme oxygenase 1 (HO-1) subsequently breaks down the pro-oxidant and pro-inflammatory heme into carbon monoxide, biliverdin, and iron. Carbon monoxide has vasodilatory, antiproliferative, anti-inflammatory, and antioxidant properties,[30-33] while biliverdin is an antioxidant that is converted by biliverdin reductase to bilirubin.[34,35] Biliverdin reductase itself has catalytic antioxidant properties.[34] The heme-derived oxidant iron is directly sequestered and inactivated by ferritin.[36] Additionally, haptoglobin-hemoglobin binding to CD163 signals anti-inflammatory IL-10 and HO-1 induction in circulating monocytes.[37] Thus, the antioxidant, anticoagulant, antiproliferative, and vasodilating effects of the CD163/HO-1/biliverdin reductase systems likely represent an evolved compensation for the nitric oxide scavenging, vasoconstrictive, proliferative, inflammatory, and pro-oxidant effects of extracellular hemoglobin, heme, and heme-iron.

When the capacity of these scavenging mechanisms has been saturated during acute or chronic hemolysis, levels of hemoglobin and heme increase in the plasma and urine. Plasma hemoglobin has the ability to scavenge nitric oxide while heme possesses multiple proinflammatory and pro-oxidant properties.

Hemolysis Causes Local and Systemic Nitric Oxide Deficiency Through the Release of Hemoglobin in Plasma

Nitric oxide reacts with hemoglobin in an extremely fast and irreversible reaction ($10^7\ M^{-1}s^{-1}$) that produces an inactive oxidation product nitrate (NO_3) and methemoglobin.[6] The speed and irreversibility of this reaction is such that very little hemoglobin can completely inhibit endothelial nitric oxide and produce endothelial dysfunction. For example, 0.01 g/dL of hemoglobin is sufficient to completely inhibit aortic ring dilation on exposure to acetylcholine.[38] Under normal physiological conditions, the reaction rate of nitric oxide and hemoglobin is severely limited by approximately 600-fold due to multiple diffusional barriers to nitric oxide around the RBC membrane and along

the endothelium in laminar flowing blood.[7-9] According to this model, vascular homeostasis is dependent on the compartmentalization or physical separation of hemoglobin from endothelium.[5] During intravascular hemolysis, this separation and the diffusional barriers are disrupted, resulting in efficient nitric oxide scavenging and endothelial dysfunction.[39,40]

Haptoglobin can bind approximately 0.07 to 0.15 g/dL of hemoglobin depending on the haptoglobin allotype.[41] Once the capacity of this hemoglobin-scavenging protein is exceeded, consumption of endogenous nitric oxide intensifies. Plasma hemoglobin levels in patients with PNH are commonly in the range of 0.05 to 0.2 g/dL and can exceed 1.0 g/dL during severe hemolytic episodes.[42] Similarly, plasma hemoglobin levels range from 0.001 to 0.033 g/dL in SCD and can exceed 0.041 g/dL during vaso-occlusive crisis.[39,43] Because the haptoglobin/hemoglobin complex is degraded in the liver, the occurrence of steady state intravascular hemolysis in diseases such as PNH and SCD typically generates sufficient plasma hemoglobin to completely deplete haptoglobin. It has been shown that quantities of plasma hemoglobin greater than 0.01 g/dL can potently inhibit nitric oxide–dependent vasodilation in vivo.[39,44]

In vitro consumption of nitric oxide in patients with SCD is highly correlated with measured plasma hemoglobin levels ($R = 0.92$), and immunodepletion of hemoglobin from plasma eliminates the ability of the plasma to consume nitric oxide.[39] Consistent with an in vivo effect of elevated circulating plasma hemoglobin on endothelial function, patients with SCD exhibit blunted vasodilatory responses to infusions of the direct-acting nitric oxide donor sodium nitroprusside.[39] In fact, patients with plasma hemoglobin levels higher than 0.01 g/dL have an 80% reduction in nitric oxide–dependent blood flow responses. Moreover, nitroglycerin-induced vasodilation is impaired in SCD patients,[45] and diminished vasomotor response to nitric oxide donors is observed in transgenic sickle-cell mice.[46,47] These mice also exhibit systemic resistance to nitric oxide donors, which correlates with hemolytic rate and plasma hemoglobin levels.[48]

While there is clearly a state of nitric oxide–mediated resistance in SCD patients and transgenic sickle-cell mouse models, overall blood flow and cardiac output are high in the face of the associated anemia. This increase in cardiac output in response to anemia appears to be mediated by up-regulation of non–nitric oxide vasodilators such as prostacyclin and endothelium-derived hyperpolarizing factor. This is illustrated in SCD patients by normal or high basal blood flow responses to acetylcholine, an endothelium-dependent vasodilator, which is not affected by simultaneous nitric oxide synthase inhibition (L-NMMA).[45,49] The hypothesis of a compensatory increase in non–nitric oxide vasodilators was recently confirmed by Kaul and colleagues who demonstrated that transgenic mice expressing exclusively human hemoglobin S exhibit complete resistance to nitric oxide–mediated vasodilation accompanied by an increase in cyclooxygenase 2 (COX-2) levels and non–nitric oxide-dependent blood flow.[48]

In addition to hemoglobin decompartmentalization and nitric oxide scavenging, hemolysis also releases erythrocyte arginase, an enzyme that converts L-arginine, the substrate for nitric oxide synthesis, to ornithine, thereby further reducing the systemic availability of nitric oxide.[50-52] Consistent with this observation, the arginine-to-ornithine ratio decreases significantly as pulmonary pressures increase in SCD patients.[22]

The biological effects of nitric oxide and its removal during intravascular hemolysis are depicted in the FIGURE. We propose that the release of hemoglobin during intravascular hemolysis results in excessive consumption of nitric oxide, subsequent reduction in guanylate cyclase activity, smooth muscle contraction, vasoconstriction, and platelet activation/aggregation.

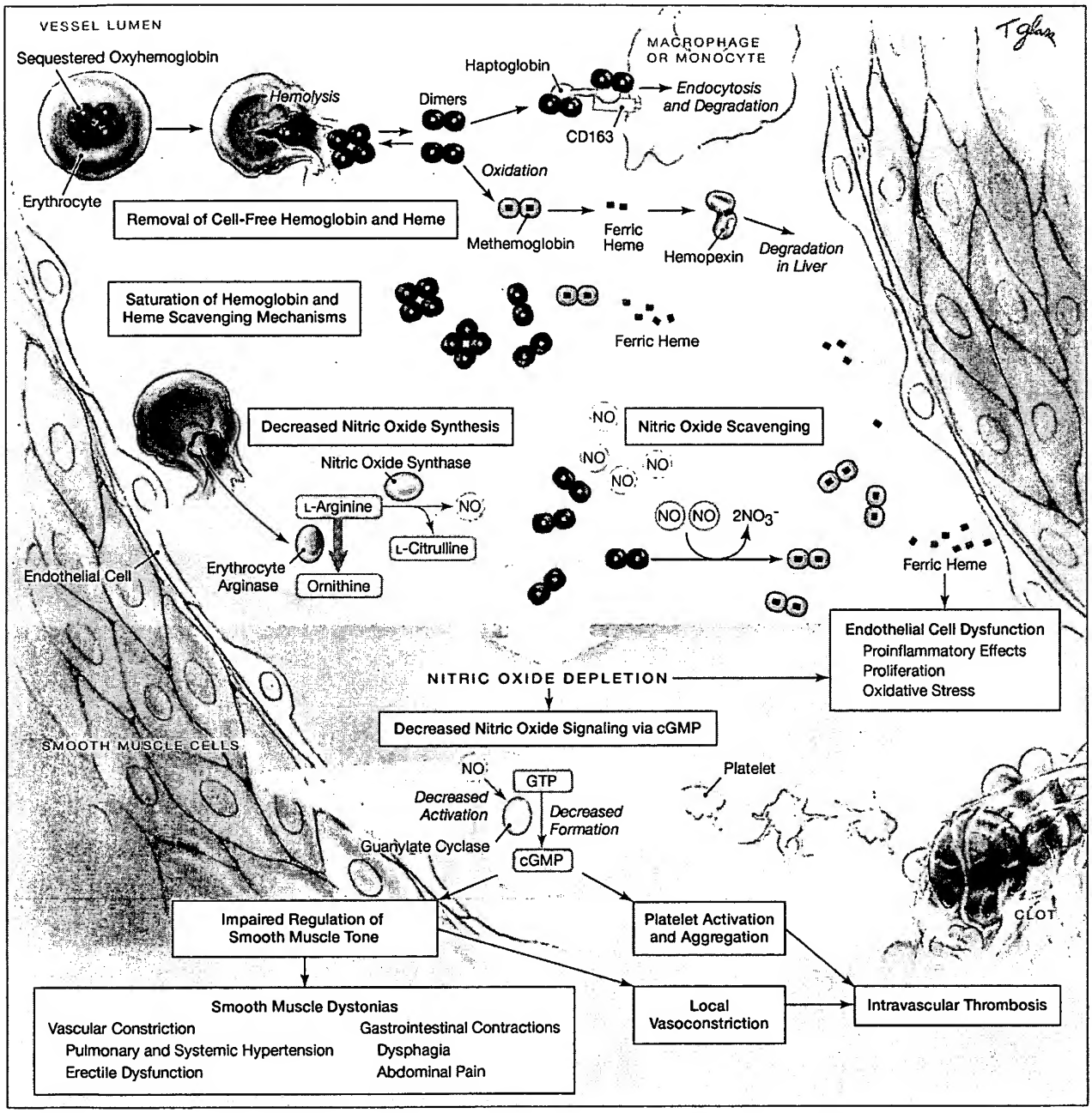
The systemic removal of nitric oxide has been shown to contribute to clinical morbidities, including severe esophageal spasm and dysphagia, abdominal pain, erectile dysfunction, and thrombosis.[16,17,23-26] In addition, systemic release of hemoglobin is associated with pulmonary and systemic hypertension,[17,20,53-55] decreased organ perfusion, and increased mortality.[53-58] Plasma hemoglobin and its breakdown product heme can also directly activate endothelial cells and further promote inflammation and coagulation.[27]

Plasma Hemoglobin Causes Dose-Dependent Increases in Adverse Clinical Signs and Symptoms

Stroma-free hemoglobin, cross-linked human or bovine hemoglobin, purified human hemoglobin, or recombinant human hemoglobin preparations have been administered to patients, human volunteers, and animals. As summarized in the TABLE, intravascular plasma hemoglobin is associated with a dose-dependent increase in adverse clinical signs and symptoms, including hemoglobinuria, abdominal pain, sternal pain, esophageal spasm, and dysphagia, as well as increases in blood pressure, platelet activation, creatine kinase level, and mortality.

Hemoglobinuria and Renal Dysfunction. Hemoglobinuria is one of the most prominent clinical signs of excessive intravascular hemolysis and is commonly associated with the administration of hemoglobin solutions.[11-15] Plasma hemoglobin is normally filtered through the glomerulus and actively reabsorbed in proximal tubule cells where it is catabolized with release of iron in the form of hemosiderin. When the kidney's reabsorption capacity is exceeded, clinically significant hemoglobinuria occurs. Acute renal failure may occur during severe episodes of hemoglobinuria.[59] Persistent severe hemoglobinuria is also associated with substantial proximal tubule hemosiderin deposition, Fanconi syndrome (defective renal reabsorption of small molecules leading to hyperaminoaciduria, glycosuria, hyperphosphaturia, and

Figure. Pathobiological Effects of Cell-Free Plasma Hemoglobin and Nitric Oxide (NO) Depletion During Intravascular Hemolysis



During intravascular hemolysis, hemoglobin is released into the plasma where it is normally cleared by the hemoglobin scavengers haptoglobin, CD163, and hemopexin. Haptoglobin-hemoglobin complexes bind to CD163 on the surface of macrophages/monocytes initiating endocytosis and degradation of the complex. Hemoglobin also releases ferric heme on oxidation, which is bound by hemopexin and degraded by hepatocytes in the liver. Excessive hemolysis saturates and depletes these hemoglobin removal systems and leads to a buildup of hemoglobin and heme in the plasma. Plasma hemoglobin and heme mediate direct proinflammatory, proliferative, and pro-oxidant effects on vessel endothelial cells. NO is normally generated from L-arginine in vessel endothelial cells by the enzyme nitric oxide synthase (NOS). NO maintains smooth muscle relaxation and inhibits platelet activation and aggregation, thereby regulating vessel tone and promoting organ system homeostasis. During intravascular hemolysis, NO availability can be severely limited by its reaction with oxyhemoglobin (NO scavenging) and by the breakdown of the substrate for NO synthesis, L-arginine, by the red cell enzyme arginase, despite elevated levels of NOS (decreased NO synthesis). NO depletion results in decreased activation of guanylate cyclase, an enzyme required for the generation of cyclic guanine monophosphate (cGMP). Decreased cGMP levels disrupt regulation of smooth muscle tone resulting in dystonias, including systemic and pulmonary hypertension, erectile dysfunction, dysphagia, and abdominal pain. Decreased cGMP levels through the depletion of NO can also lead to platelet activation and aggregation, promoting clot formation. GTP indicates guanosine 5'-triphosphate.

bicarbonate and water loss), and chronic renal failure.[59,60]

Gastrointestinal Dystonias and Pain. Administration of hemoglobin preparations to healthy human volunteers is associated with dose-dependent gastrointestinal symptoms, including abdominal pain, esophageal spasms, and dysphagia.[11,12,14,16,17] Increasing doses of recombinant hemoglobin result in increases in the duration of esophageal contractions.[16] Further, acute episodes of intravascular hemolysis in patients undergoing long-term dialysis with plasma hemoglobin levels ranging from approximately 0.3 to 2.1 g/dL have also been associated with abdominal pain.[61]

Hemoglobin-induced esophageal spasms are most likely attributable to nitric oxide consumption, as inhibition of this molecule in healthy human volunteers results in an increase in esophageal peristaltic amplitude and velocity (spasms) and a decrease in gastric distention-triggered transient lower esophageal sphincter relaxation.[62] Consistent with this hypothesis, augmentation of the downstream effect of nitric oxide via inhibition of phosphodiesterase type 5 (PDE5) with sildenafil relieves spasms in patients with esophageal motor disorders.[63,64]

Vasoconstriction and Systemic and Pulmonary Hypertension. The administration of cell-free hemoglobin solutions to healthy volunteers and patients is commonly associated with a dose-dependent increase in systolic and diastolic blood pressure,[11-15,17,18] which is reversed by the administration of the nitric oxide donor, sodium nitroprusside, confirming the importance of nitric oxide scavenging in vasoregulation.[19] Plasma hemoglobin and erythrocytes also augment hypoxic pulmonary vasoconstriction by scavenging nitric oxide, with plasma hemoglobin demonstrating an approximate 1000-fold greater nitric oxide scavenging potency in these models.[20]

Pulmonary arterial hypertension is an increasingly recognized complication of chronic hereditary and acquired hemolytic anemias, including SCD,[22,51,65-69] thalassemia intermedia and major,[70-76] PNH,[77,78] hereditary spherocytosis and stomatocytosis,[79-84] microangiopathic hemolytic anemias,[85-91] and pyruvate ki-

Table. Clinical Signs and Symptoms Associated With the Administration of Hemoglobin Solutions

Source	Model	Hemoglobin Dose	Estimated Blood Hemoglobin Level* g/dL	Estimated Blood Hemoglobin Level* μM	Clinical Signs and Symptoms
Przybelski et al, 1996[17]	24 Healthy adult volunteers	25 mg/kg 50 mg/kg 100 mg/kg	0.05 0.10 0.20	7.8 15.6 31.2	Abdominal pain and sternal pain; increased CK level; increased BP with high dose
Savitsky et al, 1978[11]	10 Healthy adult men	16 g	0.23	35.9	Hemoglobinuria; abdominal pain; increased BP; creatinine clearance reduction
Sloan et al, 1999[58]	112 Patients with traumatic hemorrhagic shock	50-100 g	0.71-1.42	110.9-221.8	Increased mortality
Carmichael et al, 2000[12]	42 Healthy adult male volunteers	1.7-42 g	0.02-0.60	3.7-93.7	Hemoglobinuria moderate >0.10 g/dL; increased BP over 0.05 g/dL with increased duration over 0.10 g/dL; abdominal pain and dysphagia: <0.40 g/dL (12/32 mild); >0.40 g/dL (10/10 moderate-severe); dysphagia relieved by smooth muscle relaxants
Murray et al, 1995[16]	9 Volunteers	0.11-0.15 g/kg	0.11-0.15	17.1-23.4	Dose-dependent increase in duration of esophageal contractions; dysphagia and CP: 0.15 g/dL (4/6 participants)
Lamy et al, 2000[13]	209 Postcardiac bypass patients	25 g 50 g 75 g	0.36 0.71 1.08	56.2 110.9 168.7	Hemoglobinuria; significant increase in BP, SVR, PVR after 0.36 g/dL; increased pancreatic enzyme levels; jaundice
Viele et al, 1997[14]	48 Healthy adult male volunteers	0.015-0.32 g/kg	0.02-0.32	2.3-50.0	Hemoglobinuria; dose-dependent gastrointestinal symptoms including abdominal pain and dysphagia: 0.02-0.11 g/dL (6/16 participants), 0.15-0.22 g/dL (11/12 participants), 0.25-0.32 g/dL (6/6 participants); prophylactic smooth muscle relaxants required in 2 highest dose groups; increased amylase/lipase levels; transient modest increase in BP and heart rate
Lamuraglia et al, 2000[18]	72 Vascular surgery patients	60 g	0.86	134.3	Significant increases in BP and BUN; trends for increases in creatinine and amylase levels
Saxena et al, 1999[15]	85 Stroke patients	25 mg/kg 50 mg/kg 100 mg/kg	0.03 0.05 0.10	3.9 7.8 15.6	Hemoglobinuria; jaundice at 0.10 g/dL; dose-dependent increase in BP duration
Olsen et al, 1996[25]	66 Rats CEA model	0.88 g/kg	0.62	96.8	71% Increased platelet activation; 26% increase in BP

Abbreviations: BP, blood pressure; BUN, blood urea nitrogen; CEA, carotid endarterectomy; CK, creatine kinase; CP, chest pain; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance.
*All blood hemoglobin values are estimates except for those of Przybelski et al,[17] which reflect actual plasma values; calculations assume a weight of 70 kg and a blood volume of 7 L.

nase deficiency.[92] There are a number of pathophysiological features shared by these disparate disorders, including intravascular hemolysis, iron overload, a propensity toward thrombosis, and surgical or autosplenectomy.

In a recent study of 195 adult patients with SCD, 32% had mild to severe pulmonary hypertension. Markers of hemolysis, including anemia, bilirubin, lactate dehydrogenase (LDH), and aspartate aminotransferase (but not liver-specific alanine aminotransferase), were associated with pulmonary hypertension.[22] In addition to limiting nitric oxide bioavailability via hemoglobin-based nitric oxide scavenging and dysregulated arginine metabolism, hemolysis is associated with activation of downstream adhesion, prothrombotic, and pro-oxidant pathways that may further contribute to endothelial dysfunction and vasculopathy.[39,49,93,94] Other mechanisms may also contribute to the development of pulmonary hypertension, including chronic thromboembolism and in situ thrombosis, asplenia, pulmonary fibrosis, liver cirrhosis secondary to iron overload and hepatitis C, and induction of hypoxia-inducible factor 1α–dependent factors such as vascular endothelial growth factor, endothelin 1, and erythropoietin.[22,69,76,84,95,96]

Thrombosis and Platelet Activation. Excessive plasma hemoglobin may contribute to platelet activation and thrombosis. The infusion of cross-linked hemoglobin increases platelet aggregation and adhesion in vivo on prothrombotic surfaces such as an injured vessel wall.[25] Additionally, administration of heme in healthy volunteers is associated with thrombophlebitis, demonstrating that heme can cause vascular inflammation followed by vascular obstruction in vivo.[97] Interestingly, the addition of cell-free hemoglobin to human serum at concentrations of 0.2 to 2.0 g/dL causes a dose-dependent inhibition of the metalloprotease ADAMTS13, an enzyme critical in limiting platelet thrombus formation.[98]

The major untoward effects of plasma hemoglobin on platelet function are most likely mediated by the scavenging of nitric oxide. Nitric oxide has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets, and inhibit platelet adhesion through increasing cyclic guanine monophosphate (cGMP) levels.[24,99] In fact, nitric oxide donor drugs (S-nitrothiols) that increase systemic levels of nitric oxide have been shown to inhibit platelet aggregation.[100] Conversely, nitric oxide scavenging by hemoglobin or the reduction of nitric oxide generation by the inhibition of arginine metabolism results in an increase in platelet aggregation.[25,26,101]

Nitric oxide interacts with components of the coagulation cascade to downregulate clot formation. For example, nitric oxide has been shown to chemically modify and inhibit factor XIII, which suggests that nitric oxide deficiency would enhance clot stability and reduce clot dissolution.[102] In animal models, reduction of nitric oxide causes increases in fibrin split products and thrombin-antithrombin complexes leading to significant fibrin deposition and thrombus formation.[103] Moreover, in a patient with L-arginine deficiency, reduced nitric oxide production is associated with increased thrombin-antithrombin complexes and fibrin split products, while reversal of nitric oxide deficiency with L-arginine causes a reduction in intravascular coagulopathy.[104]

Erectile Dysfunction. Hemoglobin release during intravascular hemolysis has been implicated in the pathogenesis of erectile dysfunction in patients with PNH, presumably through the scavenging of nitric oxide.[23,60] It has been well established that local nitric oxide deficiency due to decreased synthesis, impaired release, or premature destruction is one of the factors responsible for erectile dysfunction. The capacity of PDE5 inhibitors such as sildenafil to improve erectile dysfunction via the accumulation of cGMP is dependent on the availability of nitric oxide.[105,106]

Inflammation and Oxidation. Plasma hemoglobin and heme may possess inflammatory properties. The presence of large amounts of vascular heme results in inflammatory infiltrates in various organs in mice and induction of neutrophil activation and migration in vitro.[27,107] Heme stimulates the expression of the adhesion molecules ICAM-1 (intracellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule 1), and E-selectin on endothelial cells in vitro.[108] Heme and hemoglobin blood substitutes are associated with significant increases in vascular permeability.[109,110] Plasma hemoglobin promotes formation of the biologically hazardous hydroxyl-radical, a process that may be regulated by the hemoglobin scavenger haptoglobin.[94]

Many of the proinflammatory effects of plasma hemoglobin and heme may involve consumption of nitric oxide. Studies have shown that nitric oxide inhibits cytokine-induced induction of VCAM-1, ELAM-1 (endothelial leukocyte adhesion molecule 1), and ICAM-1 resulting in an anti-inflammatory effect.[111,112] The consumption of nitric oxide by hemoglobin circumvents the anti-inflammatory properties of nitric oxide.

Paroxysmal Nocturnal Hemoglobinuria: a Prototypic Disease of Intravascular Hemolysis

Paroxysmal nocturnal hemoglobinuria is an acquired clonal disorder of the hemopoietic stem cell that is characterized by chronic intravascular hemolysis, as indicated by the grossly elevated levels of LDH in almost all patients. Episodes of severe intravascular hemolysis are manifest clinically by hemoglobinuria (the hallmark of PNH) and are also frequently associated with dysphagia, abdominal pain, erectile dysfunction, thrombosis, and disabling fatigue.[60]

The biochemical defect underlying PNH occurs in the synthesis of the glycosyl-phosphatidylinositol (GPI) anchor.[113,114] This glycolipid structure is the means by which many proteins are attached to the plasma membrane. Two GPI-linked proteins missing from PNH cells are the complement regulatory proteins CD55 (also called "decay-accelerating factor") and CD59 (also

called "membrane inhibitor of reactive lysis"). The lack of complement regulation on the PNH RBC surface renders these cells extremely sensitive to complement-mediated lysis resulting in systemic hemoglobin release.[115]

Lactate dehydrogenase catalyzes the reversible reduction of pyruvate to lactate by nicotinamide adenine dinucleotide. Red blood cells contain large amounts of LDH, and quantitation of total LDH and hemoglobin in osmotically lysed RBCs shows a near uniform correlation between these parameters in vitro.[116] Lactate dehydrogenase is released into the plasma during hemolysis, and levels of this enzyme are generally elevated in patients with intravascular hemolysis.[10,117]

Elevated levels of LDH are common among PNH patients due to ongoing chronic intravascular hemolysis.[10,118,119] Lactate dehydrogenase is accepted as an accurate measure of intravascular hemolytic rate in PNH patients, and resolution of hemolysis in these patients results in an immediate reduction in LDH levels to near normal values.[118]

A paroxysm, from which PNH derives its name, occurs when there is a sudden marked increase in the rate of intravascular hemolysis. During such paroxysms, LDH levels can reach more than 25 times that of normal.[118] Paroxysms can be precipitated by events such as infection, drugs, and trauma, or they can occur spontaneously.

Paroxysms are characterized by severe bouts of hemoglobinuria, and more than 90% of PNH patients exhibit hemoglobinuria at some point in the disease, with approximately 50% of patients presenting with this clinical sign.[120] During a severe paroxysm, hemoglobin filtered through the kidney can reach sufficient levels to turn the urine black. Severe hemoglobinuria typically lasts 3 to 7 days, but extended episodes can occur. The incidence of hemoglobinuria is increased in patients with a large proportion of PNH blood cells (large PNH clone), as clone size often correlates with the degree of hemolysis.[23] In addition, it is well documented that intense hemoglobinuria during a paroxysm can be associated with acute renal failure.[59,121]

As described above, many organ systems that are innervated by smooth muscle are adversely affected by administration of exogenous hemoglobin preparations, most likely due to the systemic removal of nitric oxide. Similarly, during paroxysms, PNH patients exhibit symptoms that are consistent with smooth muscle perturbation through the release of hemoglobin and nitric oxide scavenging, including abdominal pain, esophageal spasms, and erectile dysfunction.

Abdominal pain is experienced by approximately 35% of PNH patients during a paroxysm, and episodes are more common in patients with a large PNH clone.[23] Although thrombosis of the mesenteric venous tree has been implicated in recurrent episodes of abdominal pain,[122] many such cases do not show evidence of thrombosis. Further, abdominal pain usually rapidly resolves when the paroxysm abates, supporting the hypothesis that nitric oxide scavenging causes intestinal dystonia and spasm.

Esophageal spasm and dysphagia due to strong peristaltic waves are a common occurrence in PNH patients, with a reported incidence of approximately 23%.[23] Dysphagia in these patients has also been shown to be closely linked to a large PNH clone and hemolysis.[23] Similarly, episodes of dysphagia are most commonly associated with paroxysms and tend to resolve as the hemolysis subsides.[60]

Erectile dysfunction occurs in 35% of male patients with PNH and is associated with paroxysms and PNH clone size, although erectile dysfunction can persist beyond hemolytic episodes and in many cases become permanent.[23,60] Erectile dysfunction in these patients appears to improve with PDE5 inhibitor therapy, as long as macroscopic hemoglobinuria is absent, suggesting a role for nitric oxide scavenging in the pathogenesis (P.H., unpublished data, 2005).

Thrombosis is the most common cause of death in PNH patients. In a series of 80 PNH patients, 50% of PNH-related deaths were attributed to venous thrombosis, and approximately 40% of patients experienced thrombosis at some point in the disease.[123] The most frequent types of thrombosis in this study included hepatic, pulmonary, deep, cerebral, and superficial veins, and inferior vena cava. Interestingly, there is a tight correlation between thrombosis and a large PNH clone, and clone size correlates with hemolytic rates.[23,124]

As described above, nitric oxide plays an important role in the maintenance of normal platelet functions through the down-regulation of platelet aggregation and adhesion and the regulation of molecules in the coagulation cascade. Accordingly, the chronic consumption of nitric oxide by plasma hemoglobin has been implicated in the formation of clots in PNH patients. Indeed, thrombotic events such as Budd-Chiari syndrome increase in PNH patients during severe bouts of hemolysis (Wendell F. Rosse, MD, oral communication, August 6, 2003). The lack of the complement regulatory proteins CD55 and CD59 on the surface of PNH platelets, which renders these cells more sensitive to complement-mediated activation,[125,126] may also contribute to thrombotic tendency in these patients.

The relationship between intravascular hemolysis and plasma hemoglobin/nitric oxide–dependent symptoms was recently evaluated with a specific drug intervention in PNH patients. A monoclonal antibody that blocks cleavage of the complement component C5, thereby preventing complement-mediated red cell lysis, was administered to hemolytic PNH patients over a 3-month period.[118] This study demonstrated a dramatic reduction in intravascular hemolysis as LDH levels dropped from 3111 to 594 U/L (normal range, 150-480 U/L; $P=.002$). Similarly, the rate of hemoglobinuria (paroxysms) was reduced from 2.9 days per patient per month before treatment to 0.12 day per patient per month during treatment ($P<.001$). Therapeutic resolution of intravascular hemoly-

sis in these patients also resulted in a significant improvement in objectively studied quality of life measurements. Furthermore, clinical assessment of symptoms in these patients prior to drug therapy and after the resolution of hemolysis during treatment showed that symptoms attributed to smooth muscle dystonias, including abdominal pain, dysphagia, and erectile failure, were either completely resolved, or at least dramatically reduced, in most patients.[127] These improvements in quality of life and clinical symptoms occurred despite the observation that total hemoglobin levels were unchanged during treatment, owing to a reduction in hemolytic rate and transfusion requirements. These data suggest that intravascular hemolysis and hemoglobin release mediate much of the symptoms of PNH patients, and further imply that smooth muscle constriction and prothrombotic effects of nitric oxide scavenging by plasma hemoglobin may be involved.

CONCLUSIONS

Intravascular hemolysis represents a severe pathological condition in a number of vital organ systems. Importantly, in a variety of human volunteer, patient, and animal studies, the presence of cell-free plasma hemoglobin is reproducibly associated with adverse clinical signs and symptoms, including gastrointestinal, cardiovascular, pulmonary, urogenital, hematologic, and renal abnormalities. Further, use of drugs that affect systemic levels of nitric oxide has revealed a strong relationship between homeostasis of these organ systems and the availability of nitric oxide. The demonstration that plasma hemoglobin is an efficient scavenger of nitric oxide, and that hemoglobin levels characteristic of intravascular hemolytic diseases are sufficient to effectively deplete nitric oxide, strongly support a critical role of nitric oxide scavenging in many of the clinical manifestations of hemoglobin release. Indeed, inhibition of complement-mediated hemolysis in PNH results in a reduction in plasma hemoglobin level and resolution of various clinical symptoms, thus demonstrating the close association between hemoglobinemia and these symptoms.

These data support the existence of a novel mechanism of human disease, hemolysis-associated smooth muscle dystonia, vasculopathy, and endothelial dysfunction. The definitive link between cell-free plasma hemoglobin, nitric oxide consumption, and symptoms characteristic of intravascular hemolytic diseases is now a topic of intense investigation.

Financial Disclosures: Drs Rother and Bell are employees of and have equity ownership in Alexion Pharmaceuticals; they have also assigned to Alexion Pharmaceuticals their inventions made as employees of the company and have received no royalties from Alexion for those inventions. Dr Gladwin has a collaborative research and development agreement between INO Therapeutics and the Intramural Division of the National Institutes of Health. Dr Hillmen serves as a consultant to Alexion Pharmaceuticals and receives grant support from the company.

Acknowledgment: We thank Christopher Mojcik, MD, PhD, Alexion Pharmaceuticals, for intellectual input and critical review of the manuscript.

REFERENCES

1. Hausladen A, Gow AJ, Stamler JS. Nitrosative stress: metabolic pathway involving the flavohemoglobin. *Proc Natl Acad Sci U S A*. 1998;95:14100-14105.
2. Crawford MJ, Goldberg DE. Role for the Salmonella flavohemoglobin in protection from nitric oxide. *J Biol Chem*. 1998;273:12543-12547.
3. Gardner PR, Gardner AM, Martin LA, Salzman AL. Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc Natl Acad Sci U S A*. 1998;95:10378-10383.
4. Minning DM, Gow AJ, Bonaventura J, et al. Ascaris haemoglobin is a nitric oxide-activated "deoxygenase." *Nature*. 1999;401:497-502.
5. Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med*. 2003;348:1483-1485.
6. Olson JS, Foley EW, Rogge C, Tsai AL, Doyle MP, Lemon DD. NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic Biol Med*. 2004;36:685-697.
7. Coin JT, Olson JS. The rate of oxygen uptake by human red blood cells. *J Biol Chem*. 1979;254:1178-1190.
8. Liu X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster JR Jr. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem*. 1998;273:18709-18713.
9. Liao JC, Hein TW, Vaughn MW, Huang KT, Kuo L. Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc Natl Acad Sci U S A*. 1999;96:8757-8761.
10. Tabbara IA. Hemolytic anemias: diagnosis and management. *Med Clin North Am*. 1992;76:649-668.
11. Savitsky JP, Doczi J, Black J, Arnold JD. A clinical safety trial of stroma-free hemoglobin. *Clin Pharmacol Ther*. 1978;23:73-80.
12. Carmichael FJ, Ali AC, Campbell JA, et al. A phase I study of oxidized raffinose cross-linked human hemoglobin. *Crit Care Med*. 2000;28:2283-2292.
13. Lamy ML, Daily EK, Brichant JF, et al. Randomized trial of diaspirin cross-linked hemoglobin solution as an alternative to blood transfusion after cardiac surgery. The DCLHb Cardiac Surgery Trial Collaborative Group. *Anesthesiology*. 2000;92:646-656.
14. Viele MK, Weiskopf RB, Fisher D. Recombinant human hemoglobin does not affect renal function in humans: analysis of safety and pharmacokinetics. *Anesthesiology*. 1997;86:848-858.
15. Saxena R, Wijnhoud AD, Carton H, et al. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. *Stroke*. 1999;30:993-996.
16. Murray JA, Ledlow A, Launspach J, Evans D, Loveday M, Conklin JL. The effects of recombinant human hemoglobin on esophageal motor functions in humans. *Gastroenterology*. 1995;109:1241-1248.
17. Przybelski RJ, Daily EK, Kisicki JC, Mattia-Goldberg C, Bounds MJ, Colburn WA. Phase I study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution. *Crit Care Med*. 1996;24:1993-2000.
18. Lamuraglia GM, O'Hara PJ, Baker WH, et al. The reduction of the allogenic transfusion requirement in aortic surgery with a hemoglobin-based solution. *J Vasc Surg*. 2000;31:299-308.
19. Erhart SM, Cole DJ, Patel PM, Drummond JC, Burhop KE. Effect of alpha-alpha diaspirin crosslinked hemoglobin (DCLHb) on the potency of sodium nitroprusside and nitroglycerine to decrease blood pressure in rats: a dose-response study. *Artif Cells Blood Substit Immobil Biotechnol*. 2000;28:385-396.
20. Deem S, Kim JU, Manjula BN, et al. Effects of S-nitrosation and cross-linking of hemoglobin on hypoxic pulmonary vasoconstriction in isolated rat lungs. *Circ Res*. 2002;91:626-632.
21. Cannon RO III, Schechter AN, Panza JA, et al. Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery. *J Clin Invest*. 2001;108:279-287.
22. Gladwin MT, Sachdev V, Jison ML, et al. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. *N Engl J Med*. 2004;350:886-895.
23. Moyo VM, Mukhina GL, Garrett ES, Brodsky RA. Natural history of paroxysmal nocturnal haemoglobinuria using modern diagnostic assays. *Br J Haematol*. 2004;126:133-138.
24. Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*. 1987;2:1057-1058.
25. Olsen SB, Tang DB, Jackson MR, Gomez ER, Ayala B, Alving BM. Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model. *Circulation*. 1996;93:327-332.
26. Schafer A, Wiesmann F, Neubauer S, Eigenthaler M, Bauersachs J, Channon KM. Rapid regulation of platelet activation in vivo by nitric oxide. *Circulation*. 2004;109:1819-1822.
27. Wagener FA, Eggert A, Boerman OC, et al. Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. *Blood*. 2001;98:1802-1811.
28. Nagel RL, Gibson QH. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J Biol Chem*. 1971;246:69-73.
29. Kristiansen M, Graversen JH, Jacobsen C, et al. Identification of the haemoglobin scavenger receptor. *Nature*. 2001;409:198-201.
30. Jison ML, Munson PJ, Barb JJ, et al. Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. *Blood*. 2004;104:270-280.
31. Otterbein LE, Zuckerbraun BS, Haga M, et al. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. *Nat Med*. 2003;9:183-190.

32. Prestera T, Talalay P, Alam J, Ahn YI, Lee PJ, Choi AM. Parallel induction of heme oxygenase-1 and chemoprotective phase 2 enzymes by electrophiles and antioxidants: regulation by upstream antioxidant-responsive elements (ARE). *Mol Med*. 1995;1:827-837.
33. Ryter SW, Otterbein LE, Morse D, Choi AM. Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. *Mol Cell Biochem*. 2002;234-235:249-263.
34. Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A*. 2002;99:16093-16098.
35. Sedlak TW, Snyder SH. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics*. 2004;113:1776-1782.
36. Sears DA. Disposal of plasma heme in normal man and patients with intravascular hemolysis. *J Clin Invest*. 1970;49:5-14.
37. Philippidis P, Mason JC, Evans BJ, et al. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. *Circ Res*. 2004;94:119-126.
38. Nakai K, Ohta T, Sakuma I, et al. Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. *J Cardiovasc Pharmacol*. 1996;28:115-123.
39. Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8:1383-1389.
40. Gladwin MT, Lancaster JR, Freeman BA, Schechter AN. Nitric oxide's reactions with hemoglobin: a view through the SNO-storm. *Nat Med*. 2003;9:496-500.
41. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem*. 1996;42:1589-1600.
42. Hartmann RC, Jenkins DE Jr, McKee LC, Heyssel RM. Paroxysmal nocturnal hemoglobinuria: clinical and laboratory studies relating to iron metabolism and therapy with androgen and iron. *Medicine*. 1966;45:331-363.
43. Naumann HN, Diggs LW, Barreras L, Williams BJ. Plasma hemoglobin and hemoglobin fractions in sickle cell crisis. *Am J Clin Pathol*. 1971;56:137-147.
44. Pohl U, Lamontagne D. Impaired tissue perfusion after inhibition of endothelium-derived nitric oxide. *Basic Res Cardiol*. 1991;86(suppl 2):97-105.
45. Eberhardt RT, McMahon L, Duffy SJ, et al. Sickle cell anemia is associated with reduced nitric oxide bioactivity in peripheral conduit and resistance vessels. *Am J Hematol*. 2003;74:104-111.
46. Nath KA, Shah V, Haggard JJ, et al. Mechanisms of vascular instability in a transgenic mouse model of sickle cell disease. *Am J Physiol Regul Integr Comp Physiol*. 2000;279:R1949-R1955.
47. Kaul DK, Liu XD, Fabry ME, Nagel RL. Impaired nitric oxide-mediated vasodilation in transgenic sickle mouse. *Am J Physiol Heart Circ Physiol*. 2000;278:H1799-H1806.
48. Kaul DK, Liu XD, Chang HY, Nagel RL, Fabry ME. Effect of fetal hemoglobin on microvascular regulation in sickle transgenic-knockout mice. *J Clin Invest*. 2004;114:1136-1145.
49. Gladwin MT, Schechter AN, Ognibene FP, et al. Divergent nitric oxide bioavailability in men and women with sickle cell disease. *Circulation*. 2003;107:271-278.
50. Schnog JJ, Jager EH, van der Dijs FP, et al. Evidence for a metabolic shift of arginine metabolism in sickle cell disease. *Ann Hematol*. 2004;83:371-375.
51. Morris CR, Morris SM Jr, Hagar W, et al. Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? *Am J Respir Crit Care Med*. 2003;168:63-69.
52. Azizi E, Dror Y, Wallis K. Arginase activity in erythrocytes of healthy and ill children. *Clin Chim Acta*. 1970;28:391-396.
53. Hess JR, MacDonald VW, Brinkley WW. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. *J Appl Physiol*. 1993;74:1769-1778.
54. Lee R, Neya K, Svizzero TA, Vlahakes GJ. Limitations of the efficacy of hemoglobin-based oxygen-carrying solutions. *J Appl Physiol*. 1995;79:236-242.
55. de Figueiredo LF, Mathru M, Solanki D, MacDonald VW, Hess J, Kramer GC. Pulmonary hypertension and systemic vasoconstriction may offset the benefits of acellular hemoglobin blood substitutes. *J Trauma*. 1997;42:847-854.
56. Vogel WM, Dennis RC, Cassidy G, Apstein CS, Valeri CR. Coronary constrictor effect of stroma-free hemoglobin solutions. *Am J Physiol*. 1986;251:H413-H420.
57. Ulatowski JA, Nishikawa T, Matheson-Urbaitis B, Bucci E, Traystman RJ, Koehler RC. Regional blood flow alterations after bovine fumaryl beta beta-crosslinked hemoglobin transfusion and nitric oxide synthase inhibition. *Crit Care Med*. 1996;24:558-565.
58. Sloan EP, Koenigsberg M, Gens D, et al. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial. *JAMA*. 1999;282:1857-1864.
59. Clark DA, Butler SA, Braren V, Hartmann RC, Jenkins DE Jr. The kidneys in paroxysmal nocturnal hemoglobinuria. *Blood*. 1981;57:83-89.
60. Rosse WF. Paroxysmal nocturnal hemoglobinuria. In: Hoffman R, Benz EJ Jr, Shattil SJ, et al. *Hematology: Basic Principles and Practice*. 3rd ed. New York, NY: Churchill Livingstone; 2000:331-342.
61. Gault MH, Duffett S, Purchase L, Murphy J. Hemodialysis intravascular hemolysis and kinked blood lines. *Nephron*. 1992;62:267-271.
62. Hirsch DP, Holloway RH, Tytgat GN, Boeckxstaens GE. Involvement of nitric oxide in human transient lower esophageal sphincter relaxations and esophageal primary peristalsis. *Gastroenterology*. 1998;115:1374-1380.
63. Eherer AJ, Schwetz I, Hammer HF, et al. Effect of sildenafil on oesophageal motor function in healthy subjects and patients with oesophageal motor disorders. *Gut*. 2002;50:758-764.
64. Bortolotti M, Pandolfo N, Giovannini M, Mari C, Miglioli M. Effect of sildenafil on hypertensive lower oesophageal sphincter. *Eur J Clin Invest*. 2002;32:682-685.
65. Norris SL, Johnson C, Haywood LJ. Left ventricular filling pressure in sickle cell anemia. *J Assoc Acad Minor Phys*. 1992;3:20-23.
66. Sutton LL, Castro O, Cross DJ, Spencer JE, Lewis JF. Pulmonary hypertension in sickle cell disease. *Am J Cardiol*. 1994;74:626-628.
67. Adedeji MO, Cespedes J, Allen K, Subramony C, Hughson MD. Pulmonary thrombotic arteriopathy in patients with sickle cell disease. *Arch Pathol Lab Med*. 2001;125:1436-1441.
68. Castro O, Hoque M, Brown BD. Pulmonary hypertension in sickle cell disease: cardiac catheterization results and survival. *Blood*. 2003;101:1257-1261.
69. Vichinsky EP. Pulmonary hypertension in sickle cell disease. *N Engl J Med*. 2004;350:857-859.
70. Grisaru D, Rachmilewitz EA, Mosseri M, et al. Cardiopulmonary assessment in beta-thalassemia major. *Chest*. 1990;98:1138-1142.
71. Du ZD, Roguin N, Milgram E, Saab K, Koren A. Pulmonary hypertension in patients with thalassemia major. *Am Heart J*. 1997;134:532-537.
72. Aessopos A, Farmakis D, Karagiorga M, et al. Cardiac involvement in thalassemia intermedia: a multicenter study. *Blood*. 2001;97:3411-3416.
73. Zakynthinos E, Vassilakopoulos T, Kaltsas P, et al. Pulmonary hypertension, interstitial lung fibrosis, and lung iron deposition in thalassaemia major. *Thorax*. 2001;56:737-739.
74. Hahalis G, Manolis AS, Apostolopoulos D, Alexopoulos D, Vagenakis AG, Zoumbos NC. Right ventricular cardiomyopathy in beta-thalassaemia major. *Eur Heart J*. 2002;23:147-156.
75. Littera R, La Nasa G, Derchi G, Cappellini MD, Chang CY, Contu L. Long-term treatment with sildenafil in a thalassemic patient with pulmonary hypertension. *Blood*. 2002;100:1516-1517.
76. Atichartakarn V, Likittanasombat K, Chuncharunee S, et al. Pulmonary arterial hypertension in previously splenectomized patients with beta-thalassemic disorders. *Int J Hematol*. 2003;78:139-145.
77. Uchida T, Miyake T, Matsuno M, et al. Fatal pulmonary thromboembolism in a patient with paroxysmal nocturnal hemoglobinuria. *Rinsho Ketsueki*. 1998;39:150-152.
78. Heller PG, Grinberg AR, Lencioni M, Molina MM, Roncoroni AJ. Pulmonary hypertension in paroxysmal nocturnal hemoglobinuria. *Chest*. 1992;102:642-643.
79. Stewart GW, Amess JA, Eber SW, et al. Thromboembolic disease after splenectomy for hereditary stomatocytosis. *Br J Haematol*. 1996;93:303-310.
80. Verresen D, De Backer W, Van Meerbeeck J, Neetens I, Van Marck E, Vermeire P. Spherocytosis and pulmonary hypertension coincidental occurrence or causal relationship? *Eur Respir J*. 1991;4:629-631.
81. Hayag-Barin JE, Smith RE, Tucker FC Jr. Hereditary spherocytosis, thrombocytosis, and chronic pulmonary emboli: a case report and review of the literature. *Am J Hematol*. 1998;57:82-84.
82. Murali B, Drain A, Seller D, Dunning J, Vuylsteke A. Pulmonary thromboendarterectomy in a case of hereditary stomatocytosis. *Br J Anaesth*. 2003;91:739-741.
83. Jardine DL, Laing AD. Delayed pulmonary hypertension following splenectomy for congenital spherocytosis. *Intern Med J*. 2004;34:214-216.
84. Jais X, Till SJ, Cynober T, et al. An extreme consequence of splenectomy in dehydrated hereditary stomatocytosis: gradual thrombo-embolic pulmonary hypertension and lung-heart transplantation. *Hemoglobin*. 2003;27:139-147.
85. Stuard ID, Heusinkveld RS, Moss AJ. Microangiopathic hemolytic anemia and thrombocytopenia in primary pulmonary hypertension. *N Engl J Med*. 1972;287:869-870.
86. McCarthy JT, Staats BA. Pulmonary hypertension, hemolytic anemia, and renal failure: a mitomycin-associated syndrome. *Chest*. 1986;89:608-611.
87. Jubelirer SJ. Primary pulmonary hypertension: its association with microangiopathic hemolytic anemia and thrombocytopenia. *Arch Intern Med*. 1991;151:1221-1223.
88. Geissler RG, Ganser A, Hubner K, Hor G, Hoelzer D. 47-year-old patient with pulmonary hypertension, anemia and thrombocytopenia. *Internist (Berl)*. 1995;36:385-388.
89. Suzuki H, Nakasato M, Sato S, et al. Microangiopathic hemolytic anemia and thrombocytopenia in a child with atrial septal defect and pulmonary hypertension. *Tohoku J Exp Med*. 1997;181:379-384.
90. Labrune P, Zittoun J, Duvaltier I, et al. Haemolytic uraemic syndrome and pulmonary hypertension in a patient with methionine synthase deficiency. *Eur J Pediatr*. 1999;158:734-739.
91. Fischer EG, Marek JM, Morris A, Nashelsky MB. Cholesterol granulomas of the lungs associated with microangiopathic hemolytic anemia and thrombocytopenia in pulmonary hypertension. *Arch Pathol Lab Med*. 2000;124:1813-1815.
92. Chou R, DeLoughery TG. Recurrent thromboembolic disease following splenectomy for pyruvate kinase deficiency. *Am J Hematol*. 2001;67:197-199.
93. Hebbel RP. Auto-oxidation and a membrane-associated "Fenton reagent": a possible explanation

for development of membrane lesions in sickle erythrocytes. *Clin Haematol*. 1985;14:129-140.
94. Sadrzadeh SM, Graf E, Panter SS, Hallaway PE, Eaton JW. Hemoglobin: a biologic fenton reagent. *J Biol Chem*. 1984;259:14354-14356.
95. Manci EA, Culberson DE, Yang YM, et al. Causes of death in sickle cell disease: an autopsy study. *Br J Haematol*. 2003;123:359-365.
96. Jison ML, Gladwin MT. Hemolytic anemia-associated pulmonary hypertension of sickle cell disease and the nitric oxide/arginine pathway. *Am J Respir Crit Care Med*. 2003;168:3-4.
97. Simionatto CS, Cabal R, Jones RL, Galbraith RA. Thrombophlebitis and disturbed hemostasis following administration of intravenous hematin in normal volunteers. *Am J Med*. 1988;85:538-540.
98. Studt JD, Hovinga JA, Antoine G, et al. Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS13 inhibitor: in vitro inhibition of ADAMTS13 activity by hemoglobin. *Blood*. 2005;105:542-544.
99. Radomski MW, Palmer RM, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol*. 1987;92:639-646.
100. Megson IL, Sogo N, Mazzei FA, Butler AR, Walton JC, Webb DJ. Inhibition of human platelet aggregation by a novel S-nitrosothiol is abolished by haemoglobin and red blood cells in vitro: implications for anti-thrombotic therapy. *Br J Pharmacol*. 2000;131:1391-1398.
101. Broekman MJ, Eiroa AM, Marcus AJ. Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension: blockade of aggregation and secretion by an aspirin-insensitive mechanism. *Blood*. 1991;78:1033-1040.
102. Catani MV, Bernassola F, Rossi A, Melino G. Inhibition of clotting factor XIII activity by nitric oxide. *Biochem Biophys Res Commun*. 1998;249:275-278.
103. Shao J, Miyata T, Yamada K, et al. Protective role of nitric oxide in a model of thrombotic microangiopathy in rats. *J Am Soc Nephrol*. 2001;12:2088-2097.
104. Kayanoki Y, Kawata S, Yamasaki E, et al. Reduced nitric oxide production by L-arginine deficiency in lysinuric protein intolerance exacerbates intravascular coagulation. *Metabolism*. 1999;48:1136-1140.
105. Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM. Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol*. 1998;159:2164-2171.
106. Corbin JD, Francis SH, Webb DJ. Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology*. 2002;60:4-11.
107. Graca-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: implications for inflammatory processes. *Blood*. 2002;99:4160-4165.
108. Wagener FA, Feldman E, de Witte T, Abraham NG. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. *Proc Soc Exp Biol Med*. 1997;216:456-463.
109. Bignold LP. Importance of platelets in increased vascular permeability evoked by experimental haemarthrosis in synovium of the rat. *Pathology*. 1980;12:169-179.
110. Xue M, Del Bigio MR. Intracerebral injection of autologous whole blood in rats: time course of inflammation and cell death. *Neurosci Lett*. 2000;283:230-232.
111. De Caterina R, Libby P, Peng HB, et al. Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest*. 1995;96:60-68.
112. Kannan MS, Guiang S, Johnson DE. Nitric oxide: biological role and clinical uses. *Indian J Pediatr*. 1998;65:333-345.
113. Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell*. 1993;73:703-711.
114. Bessler M, Mason PJ, Hillmen P, et al. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. *EMBO J*. 1994;13:110-117.
115. Holguin MH, Fredrick LR, Bernshaw NJ, Wilcox LA, Parker CJ. Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. *J Clin Invest*. 1989;84:7-17.
116. Van Lente F, Marchand A, Galen RS. Diagnosis of hemolytic disease by electrophoresis of erythrocyte lactate dehydrogenase isoenzymes on cellulose acetate or Agarose. *Clin Chem*. 1981;27:1453-1455.
117. Ravel R. Factor deficiency anemia. In: *Clinical Laboratory Medicine: Clinical Applications of Laboratory Data*. 6th ed. St Louis, Mo: Mosby-Year Book; 1995:29-30.
118. Hillmen P, Hall C, Marsh JC, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2004;350:552-559.
119. Paquette RL, Yoshimura R, Veiseh C, Kunkel L, Gajewski J, Rosen PJ. Clinical characteristics predict response to antithymocyte globulin in paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 1997;96:92-97.
120. Ravel R. Depletion anemia. In: *Clinical Laboratory Medicine: Clinical Applications of Laboratory Data*. 6th ed. St Louis, Mo: Mosby-Year Book; 1995:54.
121. Rubin H. Paroxysmal nocturnal hemoglobinuria with renal failure. *JAMA*. 1971;215:433-436.
122. Valla D, Dhumeaux D, Babany G, et al. Hepatic vein thrombosis in paroxysmal nocturnal hemoglobinuria: a spectrum from asymptomatic occlusion of hepatic venules to fatal Budd-Chiari syndrome. *Gastroenterology*. 1987;93:569-575.
123. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 1995;333:1253-1258.
124. Hall C, Richards S, Hillmen P. Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH). *Blood*. 2003;102:3587-3591.
125. Wiedmer T, Hall SE, Ortel TL, Kane WH, Rosse WF, Sims PJ. Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. *Blood*. 1993;82:1192-1196.
126. Sims PJ, Rollins SA, Wiedmer T. Regulatory control of complement on blood platelets: modulation of platelet procoagulant responses by a membrane inhibitor of the C5b-9 complex. *J Biol Chem*. 1989;264:19228-19235.
127. Hill A, Elebute M, Marsh JC, et al. Sustained control of hemolysis and symptoms and reduced transfusion requirements over a period of 2 years in paroxysmal nocturnal hemoglobinuria (PNH) with eculizumab therapy. *Blood*. 2004;104:772a.